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# Commentary

## Probing toward atomic resolution in molecular topography

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Work representing an important milepost in the development of atomic force microscopy (AFM) of biological macromolecules, just published in these *Proceedings* (1), deserves special note and comment, both for its advanced level of sophistication and for its unique degree of accomplishment.

What distinguishes this work, in terms of its sophistication, is the quantitative comparison made between high-resolution surface topographs obtained from identical protein specimens by two completely independent methods. The specimen used in this work was a two-dimensional, crystalline protein array isolated from the surface of a bacterium, *Deinococcus radiodurans*. The surface topography of this sheet-like protein crystal was first imaged by AFM; more about this shortly. In addition, however, a high-resolution, three-dimensional reconstruction of the surface of the same specimen was produced by state-of-the-art, low-dose electron microscopy. Both of the molecular topographs exhibit a lateral resolution close to 1 nm.

What is most significant about the resulting comparison between the two topographs is the fact that, when superimposed, they show an average deviation in height of less than 0.5 nm. One is hard pressed to say which is more important: Does electron microscopy, the gold standard of resolution and accepted methodology, validate the AFM image, which might otherwise be thought to be limited by a host of effects that are still being discussed in the literature? Or does the AFM image, obtained in the wet state and with incomparable resolution in the third dimension, establish that electron crystallographic reconstructions of macromolecular surfaces are perhaps better than might have been supposed, given the relatively poor resolution in the third dimension that is an unavoidable consequence of access to only a restricted range of specimen tilt angles (2, 3)? The fact that AFM could even be considered as being a suitable tool to validate structural information obtained by electron microscopy shows just how far this new technique has come in the short time, less than 8 years, since it was first introduced (4).

Even more could be said by Karrasch *et al.* (1) about the reproducibility and validity of the current AFM images, however, by using methods developed previously in electron microscopy to align and superimpose images of individual molecules. Some variations in the height of the AFM surface topograph were found at equivalent positions in different unit cells, of course. What is so impressive in the final result is that the height variations were found to be less than 0.1 nm in some parts of the unit cell, which in retrospect can be described as areas of exceptional rigidity and crystallographic perfection, while other parts of the unit cell showed height variations approaching 0.5 nm. Since the variation in height is not uniform over the unit cell, it is natural to attribute the observed pattern to differences in flexibility of different parts of the structure itself.

A state-of-the-art innovation in specimen immobilization employed by Karrasch *et al.* is yet another novelty that distinguishes this work, and no doubt has been an essential ingredient of its success. In work described at greater length elsewhere (5), Karrasch *et al.* have shown that glass surfaces can be covalently modified with an aminotriethoxysilane, which can then be derivatized with *N*-5-azido-2-nitrobenzoyloxysuccinimide, a photosensitive crosslinker. Sample material is applied to this modified glass surface, where it becomes attached by covalent bonds when illuminated with light in the near ultraviolet. It is becoming increasingly well established that firm attachment of the specimen to the substrate is a prerequisite for high-resolution applications of AFM with biological molecules.

Firm attachment of the specimen to the substrate, and rigid connections within the plane of the specimen, would seem to be required by the fact that the force used during acquisition of the AFM image is orders of magnitude greater than the force needed to move individual domains within biological macromolecules. The smallest force reported for successful operation with biological specimens has been as little as 0.1 nN, yet even this tiny force is overwhelming in comparison to the force of $\approx$1 pN exerted by myosin molecules during the individual power strokes that underlie muscle contraction (6).

It is not so surprising, then, that elastic deformation and outright plastic deformation of the specimen are easily observed during AFM imaging. A very important study in this regard was published by Butt *et al.* (7), using the purple membrane of *Halobacterium halobium* as a test object. This specialized patch of cell membrane, made up of a two-dimensional crystalline array of a single protein (bacteriorhodopsin) and lipid, would seem to be ideal for AFM studies because it has very little variation in surface height, ensuring that resolution will not be degraded by "convolution" with the large radius of the probe tip. Even with forces kept below 1 nN, however, it was evident that the hexagonal symmetry of the lattice was markedly distorted by the frictional (transverse) force exerted when recording the AFM image. As a result, the resolution in the direction parallel to the scan was worse than the nominal 1.1-nm resolution observed in the direction perpendicular to the scan direction (7). Another example relevant to this point is found in recent images of cholera toxin, a protein of 85 kDa, stabilized within a bilayer of polymerizable phospholipid (8). In specimens prepared with just the B subunit, a high percentage of molecules show the pentagonal subunit structure and the central hole expected of this molecule when the probe force is below 1 nN, but any relationship to known molecular structure is lost at probe forces close to 1 nN. Many other accounts of image distortions (9, 10) and even structural "dissection" or cutting by the probe tip (11, 12) have been reported in the recent literature.

It is encouraging that none of these deformations and distortions seemed to be apparent in the study by Karrasch *et al.* (1) of the bacterial surface-layer protein. When the biological specimen structure is simple and the topography is almost flat, even some earlier results of AFM imaging have come surprisingly close to achieving atomic resolution. Resolution of individual methyl groups was seen on the surface of a cadmium arachidate monolayer supported on a silicon oxide substrate by polar-group interactions (13). Nearly as high resolution was also achieved in imaging individual phospholipid head groups on samples prepared by transfer of a lipid monolayer onto a hydrophobic surface prepared by covalent reaction of mica with octadecyl-

trichlorosilane (14). With superior methods of attaching specimens to a rigid substrate, it would seem there is hope that other specimen materials such as purple membrane (7), virus particles (15), and nucleic acids (16, 17) may also be imaged with higher resolution and less distortion than before. What, then, is the limit that may ultimately prove to be possible in biological AFM?

The ambition of many is to see the detailed surface structure of proteins and other macromolecules in a native, aqueous environment—preferably at atomic resolution—and to observe the changes in structure of these macromolecules that occur at different stages of their biochemical function. One imagines that AFM images could be obtained with tremendous increases in speed and ease compared with electron microscope reconstructions, x-ray crystal structures, or NMR structures. High-resolution AFM topographs would require less than microgram amounts of specimen, and they could be obtained under any imaginable solution conditions that may be appropriate. Although it is probably true that these AFM images would only begin to scratch the surface of what is knowable about function-associated changes in conformation and structure, they would still be welcomed as a tool with enormously widespread utility if this ambition could be realized.

It is an open question, however, whether the ambition of atomic-resolution images of macromolecular surfaces is in principle achievable, as limited both by physics and by practical engineering considerations. Clear-headed reasoning and analysis, as well as empirical research and development, are still required in order to define the ultimate limitations on what will be possible to accomplish. The radius of curvature of probe tips is one factor that is well recognized as being a current limitation in biological AFM, and the very large forces used (see above) is a second. As we will discuss shortly, the development of sharper tips will only increase the need to operate at lower forces, and thus the two recognized limitations in current work are not independent of each other.

In order to be able to probe closely around surface bulges and into re-entrant grooves and cavities, the radius of curvature of the tip must be appreciably smaller than that of the surface features to be imaged. Fabricating a tip that can probe around the 0.5-nm width of a surface loop of polypeptide, or down into a cleft between two domains of a folded protein, poses engineering and materials challenges for which there are yet no known solutions. We must envision here a rigid rod that can tap and probe, like a blind explorer's cane, following the molecular surface as faithfully as individual water-of-hydration molecules can do. But even if such sharply pointed tips were readily available today, the force at which the AFM is operated would have to be drastically reduced to prevent the tip from breaking through the molecular surface, inserting itself like a needle into the bulk of a protein domain. What force, as an example, would be required to thread a 0.5-nm-diameter needle between segments of protein secondary structure, such as the space along the axis of a four-helix bundle? Whatever this insertion force may be, the ultimate AFM of our ambitions must operate with a lighter touch than that. Fortunately, the limits as to how low a force can be used for stable AFM imaging have in no way yet been reached; atomic resolution images of calcite in water have recently been obtained at forces as low as $\approx$0.01 nN (18).

Operation at too sensitive—i.e., too low—a force brings new complications, however, that themselves have the potential to frustrate our desire to see surface detail at atomic resolution. If the probe position is advanced only to the point where a tiny, noninvasive force is exerted on the specimen, the probe may turn out to be so far above the molecular surface that atomic resolution is no longer possible. As an example, when the tip is not in direct steric (repulsive) contact with the organic molecule, the surfaces of constant force may be determined by hydration effects and electrostatic interactions, which are longer range (and thus lower resolution in nature) than the forces that would report atomic-resolution surface features. Confronted with this particular sour lemon, some have already found a way to turn it into sweet lemon pie by using the AFM to directly measure hydration forces and electrostatic interactions between different types of surface materials (19–21).

Special mention should be made of the recent review by Yang *et al.* (22), where a much more complete citation of important contributions will be found than the limited space here has permitted. An important threshold has now been crossed in showing that AFM surface maps compare favorably, at a resolution of $\approx$1 nm, to those which can be obtained—with far greater effort—by electron microscopy (1). Even with little or no further development in methodology and instrumentation, new opportunities for the characterization of mesoscopic conformational changes have been opened up by this important advance. While there are plenty of challenges that still lie ahead, there must also be a lot of fun yet to be had in defining precisely what is possible in a fundamental sense, and in seeking methods and designs that can get as close as possible to what physical reality will permit.


1. Karrasch, S., Hegerl, R., Hoh, J. H., Baumeister, W. & Engel, A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 836–838.
2. Baumeister, W., Barth, M., Hegerl, R., Guckenberger, R., Hahn, M. & Saxton, W. O. (1986) *J. Mol. Biol.* **187**, 241–250.
3. Glaeser, R. M., Tong, L. & Kim, S.-H. (1989) *Ultramicroscopy* **27**, 307–318.
4. Binnig, G., Quate, C. F. & Gerber, C. (1986) *Phys. Rev. Lett.* **56**, 930–933.
5. Karrasch, S., Dolder, M., Schabert, F., Ramsden, J. & Engel, A. (1993) *Biophys. J.* **65**, 2437–2446.
6. Ishijima, A., Doi, T., Sakurada, K. & Yanagida, T. (1991) *Nature (London)* **352**, 301–306.
7. Butt, H.-J., Downing, K. H. & Hansma, P. K. (1990) *Biophys. J.* **58**, 1473–1480.
8. Yang, J., Tamm, L. K., Tillack, T. W. & Shao, Z. (1993) *J. Mol. Biol.* **229**, 286–290.
9. Yang, J. & Shao, Z. (1993) *Ultramicroscopy* **50**, 157–170.
10. Vesenka, J., Manne, S., Giberson, R., Marsh, T. & Henderson, E. (1993) *Biophys. J.* **65**, 992–997.
11. Hansma, H. G., Vesenka, J., Siegerist, C., Kelderman, G., Morrett, H., Sinsheimer, R. L., Elings, V., Bustamante, C. & Hansma, P. K. (1992) *Science* **250**, 1180–1184.
12. Hoh, J. H., Sosinski, G. E., Revel, J.-P. & Hansma, P. K. (1993) *Biophys. J.* **65**, 149–163.
13. Radmacher, M., Tillmann, R. W., Fritz, M. & Gaub, H. E. (1992) *Science* **257**, 1900–1905.
14. Egger, M., Ohnesorge, F., Weisenhorn, A. L., Heyn, S. P., Drake, B., Prater, C. B., Gould, S. A. C., Hansma, P. K. & Gaub, H. E. (1990) *J. Struct. Biol.* **103**, 89–94.
15. Droz, E., Taborelli, M., Wells, T. & Descouts, P. (1993) *Biophys. J.* **65**, 1180–1187.
16. Bustamante, C., Vesenka, J., Tang, C. L., Rees, W., Guthod, M. & Keller, R. (1992) *Biochemistry* **31**, 22–26.
17. Hansma, H. G., Sinsheimer, R. L., Li, M.-Q. & Hansma, P. K. (1992) *Nucleic Acids Res.* **20**, 3585–3590.
18. Ohnesorge, F. & Binnig, G. (1993) *Science* **260**, 1451–1456.
19. Butt, H.-J. (1991) *Biophys. J.* **60**, 1438–1444.
20. Hoh, J. H., Cleveland, J. P., Prater, C. B., Revel, J.-P. & Hansma, P. K. (1992) *J. Am. Chem. Soc.* **114**, 4917–4918.
21. Tsao, Y.-H., Evans, D. F. & Wennerstrom, H. (1993) *Science* **262**, 547–550.
22. Yang, J., Tamm, L. K., Somlyo, A. P. & Shao, Z. (1993) *J. Microsc.* **171**, 183–198.